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The Enteric Network: Interactions between the Immune and Nervous Systems of the Gut

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SUMMARY

Interactions between the nervous and immune systems enable the gut to respond to the variety of dietary products that it absorbs, the broad spectrum of pathogens that it encounters, and the diverse microbiome that it harbors. The enteric nervous system (ENS) senses and reacts to the dynamic ecosystem of the gastrointestinal (GI) tract by translating chemical cues from the environment into neuronal impulses that propagate throughout the gut, and into other organs in the body including the central nervous system (CNS). This review will describe the current understanding of the anatomy and physiology of the GI tract, focusing on the ENS and the mucosal immune system. We highlight emerging literature that the ENS is essential for important aspects of microbe-induced immune responses in the gut. While most basic and applied research in neuroscience has focused on the brain, the proximity of the ENS to the immune system and its interface with the external environment suggest that novel paradigms for nervous system function await discovery.

eTOC/In Brief

Following its initial discoveries in the 1980s and 1990s, a rebirth in neuro-immunology is emerging in the scientific literature. As knowledge of the gastrointestinal tract expands, including its neuronal, immunological, and microbial constituents, in this review, Mazmanian and Yoo provide new perspectives and hypotheses regarding mucosal neuro-immunology.

INTRODUCTION

The gastrointestinal (GI) tract spans 5 meters in length and has an epithelial surface area of ~32 square meters (Helander and Fåndriks, 2014). It is the home to 70–80% of the body's immune cells (Kagnoff, 1987), over 100 million neurons (Furness et al., 2014), and up to 100,000 extrinsic nerve endings (Grundy and Brookes, 2011). The microbiome consists of as many as 40 trillion cells and at least hundreds of different species (Lozupone et al., 2012; Sender et al., 2016). An important function of the GI tract is to sense and respond to external cues. Diverse cellular interactions are responsible for interpreting them, and these

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interactions must be amenable to the change and flux in the molecular environment of the GI tract. Thus, there are countless components that necessitate GI function, and equally complex changes that can affect it. Accordingly, 70 million people in the United States (>20% of the population) are affected by digestive diseases every year (Peery et al., 2012), and GI dysfunction is often a comorbidity with numerous non-intestinal conditions. As such, interactions between interdependent, cellular pathways in the gut and the periphery may underlie processes involved in health and disease.

The GI ecosystem can be largely characterized by the exchange of molecules between and within luminal constituents and the host. Environmental and dietary molecules are necessary for host survival but, in addition, are important factors that affect gut microbes and the factors they produce. How these luminal components, as a whole, interact with the cells and molecules of the intestine elucidate complex and coordinated events that occur in the GI tract. Homeostatic communication across the intestinal epithelium involves contributions by diet and the microbiota, interacting with the mucosal immune system and the enteric nervous system (ENS). In the GI tract, multiple distinct cell types can produce a given modulatory molecule, and conversely, a given molecule is able to affect various cell types. This molecular synchrony, and deviations from it, impact expansive GI and non-GI physiologies. Yet, understanding of cellular and molecular interconnectivity at this critical interface between the body and the environment remains largely unexplored. This review will focus on the diverse cellular anatomy of the GI system, and discusses the molecules and receptors that various cell types use to communicate and function. The GI tract represents a direct portal to the molecular universe, and the unique juxtaposition of its nervous and immune systems suggests a vital role for neuro-immune interactions in the gut.

Structural Anatomy of the GI tract

Digestion, absorption, and secretion in the GI tract primarily occur in the stomach, small and large intestines. Anatomically, the intestinal tissue can generally be compartmentalized by the mesentery and serosa, muscularis, submucosa, lamina propria, epithelium, and lumen (Figure 1). Major extrinsic arteries, veins, lymphatics, and nerve fibers enter and exit the tissue through the mesentery. It also encloses the mesenteric lymph nodes (MLNs), which are the draining lymph nodes of the intestines. Immunological development and lymphocyte transport occur within the intestinal tissue, but movement to and from peripheral lymphoid tissues requires passage through the mesentery. The mesentery is contiguous with the serosa, which is the outermost layer of mesothelium that encapsulates and lubricates the GI tract so that peristaltic contractions are uninhibited. The outermost layers of intestinal tissue proper are collectively called the muscularis. This region is made up of an outer longitudinal and inner circular muscle layer. These layers are orthogonal to each other, providing stretch and shear flexibility, and are also resident to many immune cells (Kalff et al., 1998). The myenteric plexus lies between the two layers of smooth muscle and the submucosal plexus resides luminal to the muscularis. Both consist of vast networks of neurons and glia that extend throughout GI tract, sending impulses and sensing inputs to and from the various intestinal compartments including the mucosa. Vasculature extends throughout the submucosal layer, bringing within it circulating immune cells that infiltrate and exit the tissue. Immune structures such as Peyer’s patches and lymphoid follicles emanate from the

submucosa and extend into the mucosa which consists of the lamina propria and innermost epithelial layer. The lamina propria contains many innate and adaptive immune cells, but also is made of the connective tissue that is important for GI structural identity. Furthermore, vascular, neuronal, and glial processes extend throughout the lamina propria and through the mucosa, and the diverse cells and molecules associated with these structures are important for enteric function. As will be described below, distinct subtypes of intestinal epithelial cells (IECs) are responsible for absorption, communication, and protection, and these roles are mediated by diverse effector molecules that function apically and basolaterally. Finally, the lumen contains the molecules excreted and consumed by the host and the microbiome that has adapted to survive the GI tract. This structural and cellular compartmentalization, described in detail below, will provide anatomical reference for the vast neuro-immune interactions that may occur in the GI tract.

The Intestinal Epithelium

The intestinal epithelium is comprised of distinct IEC types that mediate communication between the host and the luminal environment. The four most abundant IECs are enterocytes, goblet cells, Paneth cells, and enteroendocrine cells (EECs) (Figure 3). Enterocytes are the primary absorptive cells in the epithelium and they increase their surface area with apical microvilli structures. Goblet cells are responsible for producing and secreting mucin proteins into the lumen. Mucins are heavily glycosylated proteins that not only provide a protective barrier from the lumen, but also provide a medium to facilitate molecular exchange between the epithelium and the environment (Johansson and Hansson, 2016). Paneth cells reside mainly in ileal intestinal crypts and secrete potent antimicrobial products via release of granule contents (Bevins and Salzman, 2011). As such, antimicrobial peptides (AMPs) are secreted when the epithelium senses microbe associated molecular patterns, and are downregulated in germ-free (GF) mice (mice free of all microorganisms) (Ayabe et al., 2000; Satoh et al., 1986; Vaishnava et al., 2011). Finally, EECs produce a variety of modulatory, neuroendocrine molecules. These cells have been commonly referred to as the “taste” cells of the gut (Sternini et al., 2008), as they are popularly known for their chemosensation and production of molecules that control aspects of feeding, such as appetite.

Other IECs are important for directing innate and adaptive immunological responses to luminal antigens. In particular, microfold cells (M-cells) aid in the immunological sampling of antigens across the epithelium (Lelouard et al., 2012). M-cells are also capable of transporting intact bacteria across the epithelium and to gut-associated lymphoid tissues (GALT) (Clark et al., 1998). Thus, M-cells are often found in close association with dendritic cells (DCs) on the luminal side of Peyer’s patches and lymphoid follicles. This gives immune cells prime position to induce an adaptive immune response. Finally, cup and tuft cells are not well characterized, but the latter has recently been shown to be important for inducing type 2 immune responses against parasitic helminths (Gerbe et al., 2016; Howitt et al., 2016).

Transport of molecules to and from the lumen occurs both transepithelially (through epithelial cells) and paracellularly (between epithelial cells) (Pacha, 2000). Paracellular

transport is mediated by selective cellular junctions known as tight junctions. Transepithelial transport can occur through primary or secondary active transport mechanisms- the former requires ATP and carrier proteins and the latter employs concentration gradients (Bröer, 2008). Furthermore, receptor-mediated transcytosis also occurs across the epithelium, as is the case with immunoglobulin A (Hansen et al., 1999). These transport mechanisms are vital for giving immune cells and neuronal processes in the mucosa access to diverse luminal cues. Although a variety of apical stimuli are important in mediating IEC function, as will be discussed, basolateral signals can also incite effector functions in IECs. Thus, the epithelium mediates critical barrier functions in a dynamic fashion, interacting with both the luminal (e.g., gut bacteria) and basolateral (e.g., immune and neuronal cells) compartments of the gut, to initiate and transmit bilateral signals at the interface of the host and its environment.

The Enteric Nervous System

The mammalian nervous system is divided into two arms, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS encompasses the brain and the spinal cord, and the PNS includes the ganglia which are aggregates of neural cell bodies where nerve bundles arise from, in the head, neck, and viscera. The autonomic (involuntary) nervous system, a division of the PNS, is characterized as sympathetic or parasympathetic, and the main neurotransmitters produced are the catecholamines (CCh- norepinephrine, epinephrine, and dopamine) and acetylcholine (ACh), respectively. Extrinsic connectivity from the CNS to the ENS is comprised of both sympathetic and parasympathetic nerve fibers. Upon leaving the hindbrain, the parasympathetic and sympathetic nerves can synapse directly onto the GI tract. For example, the parasympathetic vagus nerve, upon leaving the hindbrain, travels along the esophagus, through the diaphragm, and ultimately synapses onto the GI tract. Sympathetic nerves originate in the spinal column and synapse onto sympathetic visceral ganglia, such as the celiac, superior and inferior mesenteric ganglia. Both parasympathetic and sympathetic nerves can synapse directly onto the myenteric ganglia, smooth muscle, and mucosa (Hansen et al., 1999). Additionally, pelvic nerves, which originate in the spinal cord and leave via the sacral spinal nerve, innervate the distal colon and rectum. Pelvic nerves have traditionally been characterized as parasympathetic, but recent, controversial data by Espinosa-Medina *et al.* suggests that these may be sympathetic in their developmental origin (Espinosa-Medina et al., 2016). Adding a layer of complexity to the neural connectivity of the GI tract is the innervation of sympathetic ganglia by parasympathetic nerves. Finally, the intrinsic ENS is the expansive network of neurons and glia along the GI tract that can function autonomously, but are also tunable by its connectivity to extrinsic sympathetic and parasympathetic nerves (Furness and Costa, 1980). Thus, communication between the CNS and ENS is bidirectional.

Receptors on enteric neurons mediate important GI functions. Mechanoreceptors are responsive to mucosal abrasion and tension receptors are responsive to stretch. Chemoreceptors respond to various chemical stimuli in the lumen, such as pH, osmolarity, and nutrients. Furthermore, various receptors are responsible for regulating fluid exchange within the gut (Derrien et al., 2004). Subsets of neurons can generally be categorized by their connectivity. Intrinsic primary afferent neurons (IPANs) are large, multi-axonal sensory neurons that are responsible for detecting molecular and mechanical aberrations of the GI

tract. These neurons relay sensory impulses to other IPANs, interneurons, and ultimately intrinsic motor neurons that induce effector functions. IPANs comprise 10–30% of the neurons in both the myenteric and submucosal plexuses. Intrinsic intestinofugal afferent neurons (IFANs) send neuronal impulses from the GI tract to extrinsic, visceral ganglia where sympathetic impulses are sent back to the ENS to complete the reflex arc (Bevins and Salzman, 2011). Interneurons are responsible for connecting sensory and motor neurons, and thus propagate neuronal impulses. Muscle motor neurons are found along the entire GI tract in the longitudinal and circular muscle layers, and they respond to signals initiated by mechano- and tension receptors. Finally, vasodilator and secretomotor neurons manage fluid and molecular exchange between the GI vasculature, tissue, and the lumen (Ayabe et al., 2000; Satoh et al., 1986) (Figures 1 and 2).

Enteric glial cells (EGCs) are also significant components of the ENS and have been shown to be important benefactors towards mucosal health (Vaishnava et al., 2011). EGCs in the myenteric and submucosal plexuses have been shown to envelop enteric neurons but also associate with blood vessels and lymphatics (Sternini et al., 2008). As will be described in later sections, EGC-derived signaling molecules implicate the functional development of cell types in GI immunity.

Molecular Constituents of Gastrointestinal Neuro-immunity

The extensive GI lymphatic system mediates the flux of immune cells that occupy the GI tract. Cells and molecules of the innate and adaptive immune system require antigens and stimulating molecules that, as will be discussed, can come from a variety of cellular sources. The GI tract represents one of the most expansive immune organs, with all the necessary components for innate and adaptive immunity, but is unique because of the diverse factors that influence immunological development. In the subsequent sections, we will describe how classical neuro-, immuno-, and microbe- associated molecules have effects on noncanonical cellular partners, and how these interactions may underlie neuro-immune responses to gut bacteria.

Catecholamines—Catecholamines (CChs) are a class of neuroactive molecules secreted at sympathetic nerve endings. The bone marrow, thymus, spleen, and peripheral lymph nodes are innervated by sympathetic nerves (Rescigno et al., 2001), and accordingly, functional adrenergic receptors are expressed on virtually all leukocytes (Serafini et al., 2015). Thus, immunologists have long been intrigued by the role of sympathetic, catecholaminergic signaling in the immune system, and their effects have been extensively studied (Elenkov et al., 2000). Recently, Gabanyi *et al.* posited the role of sympathetic innervation of the GI tract and discovered potential mechanisms by which the ENS functions to polarize intestinal macrophages, spatially and immunologically (Gabanyi et al., 2016). In this study, intestinal macrophages, were found to be phenotypically compartmentalized in the muscularis and mucosa, putting them in close proximity to extrinsic and mucosal nerve fibers, respectively. Muscularis macrophages were found to be phenotypically similar to M2 (regulatory) macrophages, whereas those isolated in the lamina propria were similar to M1 (pro-inflammatory) macrophages. When naïve peritoneal macrophages were cultured with enteric neurospheres, they became more similar to muscularis macrophages, and this

determination was dependent upon engagement of the β-2 adrenergic receptor (P2AR) (Figure 4). Furthermore, sympathetic ganglia were activated during *Salmonella* infection, and this induced a β2AR activation-dependent expression of genes associated with muscularis macrophages. In another study, *ex vivo* catecholaminergic treatment of Peyer's patches exhibited lower rates of *Salmonella* translocation (D. R. Brown and Price, 2008), potentially mitigating infection. These studies suggest that adrenergic signaling occurs in the GI tract. However, tyrosine hydroxylase (an enzyme critical for CCh biosynthesis from tyrosine) immunoreactivity persists in the GI tract after extrinsic denervation (Z. S. Li et al., 2004), and this suggests that intrinsic, catecholaminergic synthesis and signaling may also be involved in modulating GI immune responses.

In addition to the direct effects that CChs may have in regulating immune responses, similar effects may occur by modulating their availability in the GI tract. UDP-glucuronyltransferase is an enzyme that has been studied in the liver, kidneys, brain, and GI tract (King et al., 2000) for its ability to transfer glucuronic acid onto endogenous and xenobiotic molecules, making them more hydrophilic and subject to excretion in the urine and feces. Hormones are also glucuronidated, giving them their rapid systemic effect. 90% of the dopamine found in the lumen of the GF mouse is glucuronidated, and 90% of the dopamine found in the GI tract of SPF mice is not. Colonizing GF mice with an SPF microbiota or a consortia of *Clostridia* species can reverse skewing towards glucuronidated products (Asano et al., 2012). Interestingly, microbes isolated from rodent GI tracts have been shown to have endogenous beta-glucuronidase (GUS) activity (Gadelle et al., 1985), and thus, colonizing GF mice with *E. coli* that produce functional GUS enzymes is sufficient for decreasing levels of glucuronidated CChs. Conversely, mutant *E. coli* that is deficient in GUS production cannot (Asano et al., 2012). Thus, gut microbes, and specifically, microbial GUS activity can shape the molecular activity and availability of CChs in the GI tract, and this may ultimately affect how leukocytes behave.

Remarkably, bacteria also express functional adrenergic receptors, QseC and QseE, through which epinephrine and norepinephrine regulate expression of virulence genes in enteric pathogens (Hadjifrangiskou et al., 2011; Moreira and Sperandio, 2012; Njoroge and Sperandio, 2012). Pathogenicity is diminished in mutant strains of *Citrobacter rodentium* (an enteric pathogen) that are deficient in QseC and QseE. Also, wildtype *C. rodentium* is ineffective in colonizing mice that do not express dopamine beta-hydroxylase (Moreira et al., 2016), the enzyme required for norepinephrine and epinephrine synthesis. However, whether glucuronidated CChs are ineffective in modulating virulence, or whether bacteria that produce GUS can enhance virulence in a mouse is unknown. Regardless, this intriguingly links host CCh biosynthetic pathways to bacterial infections, and may provide novel, antibiotic-independent, strategies towards mitigating intestinal bacterial infections. In all, CChs in the GI tract are impactful signaling molecules that affect cells of multiple phyla. However, the way in which the cellular source of CChs impacts its effect on the immune system remains poorly understood.

Acetylcholine—Acetylcholine (ACh) is the primary parasympathetic neurotransmitter that is released by preganglionic nerve fibers (these fibers arise from ganglia along the spinal cord) and the vagus nerve. ACh has been studied for its powerful anti-inflammatory effects

in the periphery. It was first found that vagal stimulation was sufficient in suppressing systemic inflammation in response to endotoxin (Borovikova et al., 2000). It was later discovered that endotoxemic mice deficient in α7-nicotinic acetylcholine receptor (α7-nAchR) have increased systemic levels of TNFα, IL-1β, and IL-6, and these mice could not suppress TNFα levels upon vagal stimulation. Specifically, α7-nAChR expression on macrophages was necessary for the observed ACh-mediated TNFα suppression (H. Wang et al., 2003). In another study that followed, splenic nerve stimulation produced similar inhibition of the inflammatory TNFα response to LPS (Rosas-Ballina et al., 2008). However, vagal innervation results in ACh release on the celiac ganglion which in turn sends noradrenergic signals to the spleen via the splenic nerve (Berthoud and Powley, 1993). Thus, the question remained on how vagal and splenic nerve stimulation are able to produce similar effects, when the vagus nerve does not directly innervate the spleen and the splenic nerve does not produce acetylcholine, all the while, the immunoregulatory effects appeared to function through ACh receptors. In a paradigm shifting study in neuro-immunology, ACh-producing T cells were discovered to mediate these effects. These T cells secrete ACh in response to the activation of their β-adrenergic receptors, and the resulting ACh activates α7-nAchR on macrophages to suppress TNFα production (Rosas-Ballina et al., 2011). The functional discovery of ACh-producing T cells has demonstrated the remarkable molecular reflexes at the interface of the peripheral nervous and immune systems, and such discoveries have immensely expanded the breadth and appreciation of previously known neuro-immune interactions. However, research regarding cholinergic pathways in the GI tract are only just beginning to appear in the literature. For example, it has recently been shown that specific depletion of ACh producing T cells results in reduced intestinal AMP levels and relative changes to the microbiota (Dhawan et al., 2016). Mice deficient in type 3 muscarinic ACh receptors (M_3-AChR) have a leaky intestinal barrier, higher basal levels of IFNγ, IL-17A, and TNFα, and exhibit delayed clearance of *C. rodentium* (McLean et al., 2015). Interestingly, these mice also express lower levels of IL-4 and IL-13, resulting in the delayed clearance of intestinal parasites (McLean et al., 2016).

Although the vagus and pelvic nerves have connections along the length of the GI tract, with some nerve endings directly innervating the mucosa, they are not the only source of intestinal ACh. Intrinsic neurons are also immunoreactive against choline acetyltransferase (ChAT), the rate limiting enzyme in ACh synthesis (Furness, 2012). Furthermore, these extrinsic nerve endings often synapse onto neurons in the myenteric plexus. Thus, ACh from parasympathetic nerves may induce activation of noncholinergic neurons in the ENS, and subsequent release of other neuromodulatory compounds. However, since the ENS can initiate its own neuronal reflexes independent of extrinsic inputs, it is possible that intrinsic circuitry alone can influence immune function, and similar neuro-immune interactions existing in the spleen and the periphery could also exist in the GI tract.

IECs, like immune cells, are in close proximity to neuronal projections in the mucosa. However, unlike immune cells, every IEC is in direct association with the lumen. This apical and basolateral dichotomy makes IECs an interesting and potential mediator of neuro-immune communications that occur between the mucosa and the microbiota. Specifically, ACh has been well studied for its effects on Paneth and goblet cells (Figure 3). AchR activation is important for both Paneth and goblet cell degranulation. As such, bethanechol,

an mAChR agonist stimulates degranulation (Satoh et al., 1992), while the mAChR antagonist atropine suppresses this function (Satoh, 1988). Similarly, AChR activation on goblet cells induces mucin secretion (Gustafsson et al., 2012; Birchenough et al., 2016). In mice, AChR activation also results in increased goblet-cell-associated antigen passages (GAPs), which allow goblet cells to take up luminal antigens and deliver them to DCs in the lamina propria (McDole et al., 2012). This is particularly interesting because it directly implicates ACh in the immunological sampling of luminal contents and microbes. Finally, like goblet and Paneth cells, cholinergic stimulation of EECs induces its secretion of neuroendocrine molecules (Anini and Brubaker, 2003). Ultimately, ACh is a powerful mediator of intestinal function, but again, it is unclear what the endogenous source of ACh is, and if this distinction affects its functional output on lECs, the ENS, and GI immune cells.

Neuropeptides—Neuropeptides are small protein molecules that are mainly produced by neuroendocrine cells, such as EECs (Nogueira and Barbosa, 1994). They can be used to communicate between neurons, but also have endocrine function. In the GI tract, many subtypes of EECs produce a variety of neuropeptides in response to luminal and GI cues (Gribble and Reimann, 2016; Gunawardene et al., 2011). Intriguingly, enteric neurons can have similar neurochemical profiles to EECs, and are also responsive to the neuropeptides that EECs produce (Fantaguzzi et al., 2009; Merchant, 2007). As such, the molecules glucagon like peptide 1 (GLP-1) (Amato et al., 2010) and cholecystokinin (CCK) (Ngu, 1985) are known effector molecules of EECs, but also modulate ENS activity. Furthermore, enteric neurons have been shown to “synapse” onto EECs (Bohórquez et al., 2015), and EECs have been shown to secrete their intracellular stores of effector molecules in response to membrane depolarization (Matsumura et al., 2005; Reimann et al., 2012) and heightened calcium levels (Hira et al., 2008). However, the functionality of these physical neuro-epithelial circuits is not well understood. Specifically, the secretory output of an EEC in response to an action potential from a synapsed enteric neuron is unknown.

Other GI constituents can regulate aspects of EEC neuropeptide production. For example, T cell receptor mutant mice have lower numbers of CCK producing EECs in the colon (Rubin et al., 2000), potentially linking adaptive immune function to EEC peptide production. GF mice not only have fewer EECs (Duca et al., 2012), but also have altered neuropeptide levels in the CNS (Covasa et al., 2016; Schéle et al., 2013) and GI tract (Duca et al., 2012). Furthermore, EECs express functional Toll-like receptors (TLRs) (Bogunovic et al., 2007), and treatment of mice with TLR agonists induces CCK release (Palazzo et al., 2007), but these effects may not be limited to CCK. As previously described, EECs are oriented such that apical stimuli are translated into basolateral signals (Figure 3). Thus, EECs may represent a direct means by which microbes can actively remodel the neuromodulatory environment across the epithelium, potentially changing the activity of sensory neurons in the mucosa. Given the similarity of the neuropeptide molecules that EECs and the ENS utilize to communicate, and their potential effects on the immune system, perhaps EECs are capable of translating luminal cues to the GI neuro-immune system.

Additionally, neuropeptide effects on immunological function have been well documented. Peyer’s patches are innervated (Figure 1) by intrinsic, peptidergic neurons (Vulchanova et

al., 2007), and immunoglobulin synthesis and lymphocyte proliferation in peripheral lymph nodes and GALT are affected by neuropeptide exposure (Stanisz et al., 1986). Substance P (SP) was the first example of an immunomodulatory neuropeptide, and it was found to enhance the production of IL-1, TNFα, and IL-6 in human monocytes (Lotz et al., 1988). SP also regulates intestinal ion and fluid transport across the epithelium, and these effects are abolished by blocking neural transmission with tetrodotoxin. Often, intestinal inflammation is concomitant with excessive ion and fluid secretion, leading to symptoms such as diarrhea. As such, SP is more abundant in the GI tract of patients with ulcerative colitis and Crohn's disease (Mantyh et al., 1988). Subsequent research has shown that many other neuropeptides and their associated receptors are important for immunity. For example, in macrophages, neuropeptide Y (NPY) and peptide YY (PYY) enhance phagocytosis (la Fuente et al., 1993) and mice that are deficient in the NPY receptor 1 display impaired macrophage response to endotoxin. Furthermore, these mice have fewer naïve CD4+ T cells in peripheral lymph nodes, and reduced IFNγ production following dextran sulfate sodium (DSS)-induced colitis (Wheway et al., 2005). This suggests that immune cells harbor receptors for neuropeptides, and these receptors have an important role in shaping the maturation of the immune system. In another study, Th1 and Th2-lineage polarized T cell lines were created to have T cell receptors specific for an exogenous antigen. Thus, antigen exposure should lead to the production of cytokines representative of each respective lineage. Surprisingly, T cell exposure to neuropeptides was able to induce both canonical and non-canonical production of T helper cytokines, without exposure of the antigen (Levite, 1998). These results point to the intriguing possibility of neuropeptide-mediated, immunological plasticity and innate-like function from adaptive lymphocytes. These initial studies broadened the known impact of neuropeptides, but also elucidated the complexity of their immunological effects.

In contrast to SP and NPY, vasoactive intestinal peptide (VIP) has been the best studied antiinflammatory peptide. VIP induces regulatory (Chorny et al., 2005) and tolerogenic DCs (Delgado et al., 2005b; Ganea et al., 2006), both of which are able to induce differentiation of regulatory T cells (T_{regs}) (Delgado et al., 2005a). Furthermore, VIP inhibits TGF-β1 (Sun et al., 2000) and enhances IL-10 (Delgado et al., 1999) production in murine macrophages. Also in macrophages, TLR2 and TLR4 stimulation upregulates expression of VIP receptor type 2 (Herrera et al., 2009) and conversely, VIP inhibits LPS mediated upregulation of TLRs, and suppresses macrophage differentiation (Foster et al., 2007). In the GI tract, VIP mitigates trinitrobenzene sulfonic acid (TNBS)-induced intestinal inflammation, another mouse model of colitis. In this model, VIP also inhibits TLR expression in macrophages, DCs, lymphocytes, and colon protein extracts (Abad et al., 2003; Gomariz et al., 2005). Similarly, VIP ameliorates intestinal barrier dysfunction in *C. rodentium*-induced colitis in mice (Conlin et al., 2009). Furthermore, Toxin B from the colitogenic bacterium, *Clostridium difficile*, can activate VIP producing neurons in the submucosa (Neunlist et al., 2003). However, over time, *C. difficile* infection decreases overall levels of VIP (Nassif et al., 1995), and perhaps by depleting VIP, *C. difficile* can ultimately exacerbate enteric infection. Finally, VIP-deficient mice have multiple GI abnormalities, including deficits in goblet cell secretion (Lelievre et al., 2007) which potentially compromises the protective mucus barrier in the lumen. In all, neuropeptide balance is imperative for proper immune

function and GI homeostasis, and skewing in either direction leads to physiological irregularity, potential disease pathology, and increased infection susceptibility.

Serotonin and Histamine—Serotonin (5-HT) is produced by various cell types in the GI tract and has multiple physiological roles. 5-HT was first identified for his roles in vasoconstriction and found to be stored in platelets (Rapport et al., 1948). It has also long been established that in the GI tract, mast cells and enterochromaffin cells, a specific EEC, are the major sources of 5-HT (Erspamer and Asero, 1952). Currently, enteric neurons (Gutknecht et al., 2009) and various leukocyte types, including macrophages, DCs, B and T cells, have been found to express either tryptophan hydroxylase (TPH-1/2, the rate limiting enzyme in 5-HT biosynthesis), or 5-HT transporters (SERT) which are necessary mediators of extracellular 5-HT uptake (Holder et al., 2005; Rudd et al., 2005; X. Wang, 2006). Furthermore, a variety of 5-HT receptors have been identified in lymphoid tissues (Stefulj et al., 2000), and as a result, their expression and function in a variety of immune cells have been extensively studied (Shajib and Khan, 2015). In general, serotonergic signaling in leukocytes appears to promote immune function, whether by enhancing DC-mediated T cell activation (Leon-Ponte et al., 2007; X. Wang, 2006), or affecting macrophage polarization (las Casas-Engel et al., 2013) and phagocytosis (Csaba et al., 1975).

5-HT is also one of the earliest known molecules involved in GI peristalsis (Bülbring and R. C. Y. Lin, 1958), and it does so by directly modulating ENS activity (Costa and Furness, 1979; Hillsley and Grundy, 1998). In addition to 5-HT, mast cells produce histamine which can activate IPANs (Song et al., 2015; Starodub and Wood, 2000), and expectedly, heightened levels of histamine and 5-HT correlate with increased activation of submucosal neurons. This increase in activation is dampened by 5-HT and histamine receptor antagonists (Buhner et al., 2009), and although mast cell mediated ENS activation is compelling, its significance and physiological implications are poorly understood. Interestingly, vagal stimulation initiates release of 5-HT in enterochromaffin cells, and this appears to be mediated by adrenergic receptor activation (Ahlman et al., 1976). Mast cells also express functional adrenergic receptors, and receptor binding enhances IgE mediated degranulation of 5-HT and histamine (Yamasaki et al., 1983). Furthermore, amitriptyline, a 5-HT and norepinephrine uptake inhibitor, inhibits mast cell secretion of histamine but not 5-HT (Theoharides and Bondy, 1982). This suggests that histamine secretion from mast cells can be modulated by CChs, whereas intrinsic production of 5-HT, as opposed to the reuptake of 5-HT, is needed for adrenergic receptor-mediated 5-HT secretion. These data show that similar neural paradigms observed in the cholinergic antiinflammatory pathway may also affect the levels of 5-HT and histamine in the GI tract, but similar shortcomings in mechanistic understanding persist, such as the contribution of adrenergic signals from extrinsic sympathetic nerves versus those that are produced intrinsically. Nonetheless, these results show how molecules that are traditionally associated with leukocytes are capable of modulating the ENS and vice versa, providing more evidence of complex neuro-immune interactions in the GI tract.

In a study by Yano *et al.*, indigenous, spore-forming bacteria, which are primarily *Clostridia*, were found to be sufficient in rescuing the 5-HT deficiency observed in GF mice (Yano et al., 2015), further associating the microbiota to potential neuro-immune interactions in the

GI tract. Consistent with this finding, short chain fatty acids (SCFAs), which are the fermentation products of dietary fibers metabolized by the intestinal microbiota, were also sufficient in upregulating 5-HT (Reigstad et al., 2015; Yano et al., 2015). *Clostridia*, and specifically butyrate producing strains, are important for the induction of T_{regs} (Atarashi et al., 2013; 2011; Furusawa et al., 2013) and interestingly, patients with depression have lower levels serum 5-HT and T_{regs} that express lower levels of the 5-HT1a receptor (Y. Li et al., 2010). Furthermore, anti-CD25 depletion of T_{regs} lowers 5-HT levels and leads to depression-like behaviors in mice. Additionally, *C. rodentium* infection decreases levels of 5-HT producing EECs (O'Hara et al., 2006), while T_{regs} confers protection from it (Z. Wang et al., 2014). It is possible that specific gut bacteria are capable of modulating CNS-associated phenotypes by inducing immunological changes that are mediated by neuromodulatory compounds. These types of studies would provide powerful, mechanistic understanding of how gut bacteria incite changes beyond the GI tract and implicate more global physiologies. At the moment, it is appreciated that mucosal 5-HT is an important paracrine signaling molecule in the GI tract, however, it is less understood how diverse cell types contribute to such a unified physiological response that is dependent on 5-HT.

Neurotrophic Factors—Neurotrophic factors (NTFs) are small protein molecules that have classically been studied for their roles in sustaining neuronal growth and maturation. In the GI tract, the most abundant NTFs are the glial cell-derived neurotrophic factor (GDNF) family of ligands (GFLs, which include GDNF, Neurturin, and Artemin), and they are produced by EGCs and smooth muscle cells (Bar et al., 1997; Brun et al., 2015; Han et al., 2015). However, unlike their name suggests, NTFs can modulate neuronal activity and produce effects on non-neuronal cell types. Firstly, EGCs potentially affect neurotransmission by modulating production of neuropeptides. For example, GDNF promotes enteric neuron release of NPY (Anitha et al., 2006) (Figure 2), and GDNF-, NTN-, GFRα1- (receptor for GDNF), and RET- (whose co-receptors are GFL receptors-α1–3) deficient mice have defects in stimulus evoked release of VIP and SP. These mice also display reduced GI contractibility (Gianino, 2003; Heuckeroth et al., 1999), suggesting that EGC-mediated changes in neuropeptide levels are sufficient to cause dysregulated ENS activity. As previously described, GFL-mediated modulation of neuropeptides may have downstream effects on immune cell function. This illustrates the potential indirect effects of GFLs on neuro-immune interactions in the GI tract, however, EGC derived NTFs have recently been shown to directly affect the development of specific GI immunological compartments. It was known that GFL-mediated RET signaling is necessary for proper development of Peyer's patches (Patel et al., 2012; Veiga-Fernandes et al., 2007), but in a more recent study by Ibiza et al., GFL-mediated RET signaling was found to be necessary for the functional development of IL-22 producing innate lymphoid cell type 3 (ILC3) (Ibiza et al., 2016). ILC3s are the first innate immune cells to expand in the GI tract following bacterial infection (Sonnenberg et al., 2011), and via IL-22, ILC3s have broadly protective effects on the intestinal epithelium (Liang et al., 2006; Lindemans et al., 2015; Sugimoto et al., 2008; Zheng et al., 2008). Ibiza and colleagues found that GFLs directly activate RET expressed on ILC3s, and depletion of RET diminishes intestinal IL-22 levels and exacerbates enteric inflammation (Figure 4). ILC3s were also found in close proximity to EGCs, and as previous studies also presented (Brun et al., 2013a), TLR activation induced

expression of GFLs. Importantly, glial specific depletion of MyD88 reduced levels of GFLs and IL-22, and also recapitulated inflammatory pathologies observed in mice with ILC3 specific depletion of RET (Ibiza et al., 2016). These findings are the first to show that EGCs directly sense the microbial environment to induce GFL production, and this sensing is necessary for the development of GI immunity. This seminal study elucidates mechanisms by which components of the ENS can directly shape the immunological environment in the GI tract.

EGCs and NTFs are also implicated in intestinal neuropathies and inflammatory pathologies. Ablation of EGCs results in epithelial and neuronal damage as well as severe intestinal inflammation (Bush et al., 1998). Agangliosis (a lack of enteric ganglia) occurs in mice that are deficient in GDNF (Sánchez et al., 1996) and RET (the coreceptor GDNF family receptors-α1–3) (Schuchardt et al., 1994), and also in humans with Hirschspring's disease. Furthermore, pathology of Hirschsprung's disease is often comorbid with enterocolitis (Austin, 2012; Fujimoto et al., 1988; Imamura et al., 1992; Murphy and Puri, 2005) and although the precise etiology of this colitic pathology is unknown, patients with Hirschprung's disease often display germline mutations in RET (Brooks et al., 2005) and GDNF (Angrist et al., 1996; Bar et al., 1997). In addition to producing GI pathologies similar to those found in Hirschsprung's disease, RET-deficient mice also display a marked reduction in VIP gene transcription (Heanue and Pachnis, 2016; Lelievre et al., 2007), and as previously mentioned, VIP has anti-inflammatory effects that are protective effects against chemical- and microbe-induced colitis. Thus, glial constituents may also be important in maintaining homeostatic levels of immunoregulatory neuromodulatory compounds.

Finally, EGC proliferation is linked to intestinal inflammation (Bradley et al., 1997; Ruhl et al., 2001). Accordingly, GDNF is increased during enteric parasitic infections (Starke-Buzetti and Oaks, 2008), found in intestinal biopsies from humans with colitogenic *Clostridium difficile* infection, and upregulated in humans who suffer from ulcerative colitis and Crohn's disease (Boyen et al., 2011). However, in culture, IL-1β appears to dampen EGC proliferation while IL-10 enhances it (Ruhl et al., 2001). Although this may appear contradictory, it is possible that intestinal inflammation promotes immunoregulatory functions in EGCs through proliferation and upregulation of GFLs, but persistent, hyperinflammatory responses may result in inhibition of these processes altogether, thus leading to enteric pathology and disease. In all, it is apparent that GFLs and EGCs have a significant role in maintaining intestinal immune homeostasis. Although it is possible that enteric neurons may interface EGCs and GFLs to modulate GI immunity, it is currently unknown whether these compartments are in fact, interdependent.

Cytokines—Cytokines and chemokines are the main effector molecules of immune cells. However, enteric neurons and glia are also capable of producing cytokines. As previously alluded to, EGCs express functional TLRs (Barajon et al., 2009; Ibiza et al., 2016) and upon LPS stimulation of EGCS, heightened levels of IL-1β are observed. Furthermore, ENS cultures produce TNFα and IL-6 in response to LPS, and this production is abrogated when NF-kB signaling is inhibited (Burgueño et al., 2016). Curiously, IL-6 and the IL-6 receptor promote the growth and survival of enteric neurons in culture (Schäfer et al., 2004), but just

as IL-6 has both pro- and anti-inflammatory effects in the peripheral immune system (Scheller et al., 2011), it may also have dichotomous effects on the ENS.

As detailed previously, neurotransmitters and neuropeptides alter immune function. However, reciprocally, cytokines are also capable of modulating neuronal activity. The abundance and close proximity of neuronal varicosities to immune cells should make this unsurprising, but it is important in the study of immune responses to consider the effector functions of cytokine molecules on non-immune cell types. For example, IL-1β is heightened in the intestines of inflammatory bowel diseases (IBD) patients (Ligumsky et al., 1990), and in accordance with prior discussions, IL-1β also induces SP production in the ENS (Hurst et al., 1993). Furthermore, IL-1β engagement to its cognate receptor in *ex vivo* ENS preparations suppresses electric-field stimulation (EFS)-evoked release of ACh and norepinephrine (Collins et al., 1992). IL-1β and IL6 can excite enteric secretomotor neurons (Figure 4) and this appears to occur by suppressing extrinsic sympathetic and parasympathetic nerves (Xia et al., 1999). Thus, it is possible that enteric inflammatory symptoms are not only a product of pro-inflammatory cytokines, but also a result of IL-1β mediated upregulation of SP, and subsequent increases in secretomotor activity, fluid secretion and decreased anti-inflammatory signaling from cholinergic and adrenergic neurons. This would explain many of the aforementioned phenotypes in IBD patients, including the heightened levels of SP, IL-1β, and TNFα (Xavier and Podolsky, 2007).

Short Chain Fatty Acids—Again, SCFAs are microbial metabolic products of dietary fibers, and the most studied SCFAs are butyrate, propionate, and acetate (Campbell et al., 1997; Wolin, 1981). These metabolites are sensed by the intestinal epithelium, but can also diffuse across the epithelium (Charney et al., 1998) where they can be accessed by the enteric nervous and immune systems (Figure 3). G-protein coupled receptors (GPR) 41 and 43 are activated by acetate and propionate (A. J. Brown et al., 2003; Tazoe et al., 2008), whereas GPR109A is specific for butyrate (Thangaraju et al., 2009).

lECs from mice deficient in GPR41 or GPR43 display reduced inflammatory chemokine and cytokine profiles in response to TNBS-induced colitis and *C. rodentium* infection. These mice also exhibit delayed clearance of *C. rodentium* itself (Kim et al., 2013). Furthermore GPR43 is necessary for propionate-mediated secretion of PYY and GLP-1 (Psichas et al., 2014), and selective agonists for GPR41 and GPR 43 induce GLP1 secretion from colonic crypt cultures. Additionally, purified GPR41+ cells exhibit higher expression levels for neuropeptide and neuroendocrine hormone precursors (Nøhr et al., 2013) suggesting that an association between SCFAs and the production of neuropeptides exists. Also, PYY inhibits gastrointestinal transit and GPR41 deficiency is associated with decreased PYY production (Samuel et al., 2008). Accordingly, SCFA-mediated induction of PYY inhibits in colonic motility (Cherbut et al., 1998) which suggests that SCFA mediated modulation of enteric neuronal activity exists. Intriguingly, it was found that enteric neurons in both the myenteric and submucosal plexuses express GPR41. In contrast, immune cells in the lamina propria preferentially express GPR43 (Karaki et al., 2006a; Nøhr et al., 2013). T_{regs} isolated from the colon and small intestines express high levels of GPR43 through which propionate enhances their immunosuppressive capabilities (Smith et al., 2013). Accordingly, propionate administration alleviates symptoms during experimental autoimmune encephalomyelitis, a

mouse model of multiple sclerosis, and the mitigating effects of propionate were found to be concomitant with small intestinal Tregs induction (Haghikia et al., 2015). Mucosal mast cells (Karaki et al., 2006b) and neutrophils (Le Poul et al., 2003) also express GPR43. In neutrophils, bacterial-derived SCFAs enhance chemotaxis (Eftimiadi et al., 1987) and propionate induces degranulation (Carretta et al., 2013). Activation of GPR109A reduces intestinal inflammation and colon cancer susceptibility in mice (Singh et al., 2014). In human monocytes, activation of GPR109A by nicotinic acid has anti-inflammatory effects (Digby et al., 2012), and this is further illustrated by the ability of butyrate to inhibit mast cell activation and degranulation (Diakos et al., 2006). Butyrate and butyrate-producing bacteria, as previously described, are also sufficient for inducing differentiation of T_{regs} in mice (Atarashi et al., 2013; Furusawa et al., 2013). Finally, butyrate is also capable of inducing action potentials in both submucosal and myenteric neurons of the ENS (Kunze et al., 2009; Neunlist and Dobreva, 1999), and this is correlated with higher numbers of intrinsic, cholinergic neurons of the myenteric plexus (Soret et al., 2010). This is in agreement with prior evidence showing the potent anti-inflammatory effects of ACh, and thus it is possible that ENS sensing of butyrate may be an important aspect of coordinating intrinsic, ACh-mediated neuro-immunoregulatory responses.

Microbes, and the Enteric Immune and Nervous Systems

The intestinal microbiota is the collection of microorganisms in the GI tract, and its constituents are known to be representative of the animals' diet, habitat, and even phylogenetic order (Ley et al., 2008). It has long been appreciated that the indigenous microbiota of the GI tract is important for its development and susceptibility to enteric infections (Dubos and Schaedler, 1960), but it was not until much later that researchers demonstrated how commensal bacteria have an active role in maintaining GI immune homeostasis. In 1996, it was found that anaerobic bacteria in human feces was specifically enriched in coating by immunoglobulin A (IgA) (vanderWaaij et al., 1996). A few years later, Macpherson *et al.* discovered T cell-independent mechanisms of IgA binding to commensal gut microbes, suggesting an evolutionarily innate, immunological adaptation to the intestinal microbiota (Macpherson et al., 2000). In 2001, Hooper *et al.* showed that *Bacteroides thetaiotaomicron* modulates host genes important for IEC homeostasis (Hooper et al., 2001), and in 2005, Mazmanian *et al.* discovered that polysaccharide A (PSA) from *Bacteroides fragilis* is sufficient for the development of adaptive immune responses (Mazmanian et al., 2005). These influential studies expanded the knowledge and incited curiosity towards the microbiome, but more importantly, they empirically defined homeostatic roles of the intestinal microbiota that were not previously appreciated.

In 1932, the first GF animal was created at the Laboratories of Bacteriology at the University of Notre Dame (LOBUND) by Dr. James Reyniers (Reyniers, 1932). Later, GF rats were studied and characterized in the 1950s (Orland et al., 1954) to study dental hygiene, and were later used to study microbial colonization resistance (Van der Waaij and Vries, 1971). More recently research groups have implicated the microbiota in stress, anxiety, behavioral developmental disorders, and neurological deficits (Heijtz et al., 2011; Hoban et al., 2016; Hsiao et al., 2013; Sampson et al., 2016). This has popularized hypotheses on the "gut-brain axis". However, the role of the ENS and GI-resident immune

functions are currently understudied as conduits for the gut-brain effect, and the abundance of immune cells in the GI tract and the vast connectivity of the ENS (and its direct connectivity to the CNS) make it unlikely that the axis is independent of these interactions.

The microbiota has been shown to be altered during intestinal inflammation and immunological disease. Severity of inflammation is mediated and ameliorated by pathogenic and commensal bacteria, respectively (Aas et al., 2003; Mazmanian et al., 2008). As previously mentioned, microbes or microbial products can actively change TLR expression in most cellular compartments of the GI tract, and this alters the host's ability to sense and respond to the microbiota. Antibiotic depletion of the microbiota alters TLR expression in mice, and also results in concomitant in changes to GI motility and sensitivity to ACh (Grasa et al., 2015). TLR4 mutant mice have fewer neurons in the ENS and this is also seen in MyD88 knockout and antibiotic-treated mice (Anitha et al., 2012). Furthermore, GF animals display defects in ENS morphology (Collins et al., 2014) and excitability (McVey Neufeld et al., 2013), and these deficits are reversed by colonizing them with a complex microbiota. EGC growth, maturation, and signaling is also affected by the microbiota. For example, GF mice and antibiotic-mediated depletion of the microbiota results in non-migratory glial cells that fail to extend through the mucosa and into villi structures (Kabouridis et al., 2015). Antibiotic-treatments also reduce levels of GDNF/GFRα1 and RET, and these deficiencies can be reversed by TLR2 stimulation (Brun et al., 2013b). Furthermore, TLR2-deficient mice also display lower levels of GDNF, impaired RET signaling, and heightened inflammation in response to DSS-induced colitis. Accordingly, these phenotypes can be mitigated by exogenous GDNF administration (Brun et al., 2013b). Although exact mechanisms by which microbiota modulates neural activity in the GI tract are unknown, these findings suggest that there is an active, post-developmental role of the microbiota on ENS form and function, potentially explaining disparities in their molecular output.

Lactobacilli and Bacteroides—Many *Lactobacillus* species have been studied for their immunoregulatory role. Species such as *L. rhamnosus, L. salivarius, L. reuteri, L. planatrum, L. fermentum*, and *L. casei* have been shown to induce T_{regs} and decrease levels of inflammatory cytokines, such as IFNγ and TNFα (Foligne et al., 2007; Madsen et al., 1999; Valeur et al., 2004). *Lactobacilli* have also been studied for their neuromodulatory capabilites. Rats colonized with *L. reuteuri* display lower thresholds for IPAN activation, higher frequency of action potentials (Kunze et al., 2009; Mao et al., 2013), and shorter hyperpolarizing potentials, all of which are proxies of enhanced excitability. Interestingly, in an influential study describing the "gut-brain axis", Bravo *et al*. discovered that commensal microbes modulate physiologies beyond the GI tract and influence behaviors commonly associated with affections in the CNS. Specifically, *L. rhamnosus* reduces stress-induced corticosterone levels and decreases expression of γ-aminobutyric acid (GABA) receptors in the CNS, leading to concomitant reductions in anxiety and depression-like symptoms in mice. Interestingly, these behavioral and neurochemical phenotypes were abolished following chemically-induced vagotomy (Bravo et al., 2011). In accordance, it was later shown that *L. rhamnosus* increases the rate of spontaneous firing in vagal afferent nerves (Perez-Burgos et al., 2013). Furthermore, certain strains of *L. rhamnosus* are known to produce GABA under certain conditions (Q. Lin, 2013), and *L. reuteuri* can produce GABA

from its precursor, glutamate (Barrett et al., 2012; Siragusa et al., 2007). Thus, direct production of neuroactive molecules by gut microbes also has the potential of modulating ENS activity, but this has not been specifically addressed.

The *Bacteroides* genus also have immunoregulatory effects in the GI tract, and studies have predominantly been focused on *B. thetaiotaomicron* (Kelly et al., 2004) and *B. fragilis* (Mazmanian et al., 2008). As described previously, *B. fragilis* has been studied for its role in inducing IL-10 producing FoxP3 T_{regs} (Chu et al., 2016; Round and Mazmanian, 2010). Interestingly, *B. fragilis* mono-associated mice also display enhanced IPAN excitability, and PSA was both sufficient and necessary for this phenotype (Mao et al., 2013). These data illustrate the active role that bacteria and bacterial-associated molecules have on the excitability and activity of intrinsic sensory neurons in the ENS, but whether microbe-induced ENS activation is necessary for modulating GI immunity is unknown.

Trichinella spiralis—*Trichinella spiralis* is a parasitic nematode that infects the intestines (Wright, 1979) and induces IL-1 production (Stadnyk and Kearsey, 1996). In mice, *T. spiralis* infection causes hypophagia, the decrease in food intake (McDermott et al., 2006). This infection causes upregulation of CCK and 5-HT expression in EECs, both of which are implicated in hypophagia (Burton-Freeman et al., 1999; Hayes et al., 2006). Furthermore, CCK and IL-4 induce contractions in the longitudinal muscle (Lv et al., 2010; Zhao et al., 2003), and increased contractibility of the GI tract is associated with T. spiralis expulsion (Vallance et al., 1997). Interestingly, prior *T. spiralis* infection increases sensitivity of mouse enteric neurons to T. spiralis antigens, and this heightened sensitivity is abrogated by histamine receptor antagonism (Frieling et al., 1994). IgE mediated mast cells activation also induces histamine secretion (Ishizaka et al., 1980) and enhances *T. spiralis* expulsion (Ahmad et al., 1991). Taken together, these studies put forth a potential histamine-mediated, adaptive, neuro-immune response to pathogenic microorganisms that may be important for their clearance.

PERSPECTIVE

The GI tract is positioned to sense and respond to diverse fluxes of cues– cues can be intrinsic, extrinsic, and environmental. In the viscera, nerves from the CNS and PNS synapse onto the GI tract. At the mucosa, the host readily and selectively absorbs molecules within and across the epithelium. And in the lumen, the microbiota is under constant selective pressure from factors such as diet, environmental exposure, immune, and exocrine function. This exchange of molecular information in the GI tract illustrates intestinal “reflex loops” that direct how our body may utilize the ENS to physiologically sense and respond to changes in the environment. However, mechanisms by which functional enteric circuits integrate with microbe-mediated GI immune responses are poorly understood. For example, it is largely unknown if the ENS senses the microbial environment and initiates an immune response, or whether the ENS senses immune responses to subsequently modify, amplify, or propagate those signals. It is also unknown if or how the ENS reciprocally affects the luminal environment to shape microbial communities in the gut, and whether this effect is mediated by the immune system. Further characterization of the connectivity of the ENS with itself and other cell types will help uncover the molecules in the GI tract that dictate the

body's ability to immunologically mature and adapt to the microbiota. However, to truly dissect mechanisms by which the ENS mediates, potentiates, or responds to microbe-induced GI immunological changes, spatial and temporal manipulation of the ENS is required. Accordingly, modern advances in neuroscience, such as optogenetics and chemogenetics, have recently been implemented in the context of the ENS (Chang et al., 2015; Grubišić and Gulbransen, 2017; Rakhilin et al., 2016). While discoveries of neuro-immune interactions in the GI tract indicate ENS participation in mucosal immune development (Gabanyi et al., 2016; Ibiza et al., 2016), effects of the microbiota on these immunological changes remain largely unknown. A recent study by Yissachar *et al.* utilized an intestinal organ culture system and showed that gut bacteria can modulate the neuro-immune environment of the intestines (Yissachar et al., 2017), however, it remains unknown whether neural components are sufficient or necessary for this regulation. Thus, more future studies uncoupling the effects of the ENS on GI immune function will provide the foundation to study how the microbiota affects interactions between neurological and immunological systems of the body. Furthermore, it remains an intriguing possibility that enteric neuro-immune interactions may be the preferred route by which the microbiota has more global effects on CNS-related behavior.

Communication between the microbiome and the mucosa is essential for the maintenance of intestinal homeostasis. Given the diversity and reciprocity of extracellular signaling molecules in the GI tract, the ENS has evolved over time to receive and interpret these diverse cues, and transmit them throughout the GI tract and ultimately, the entire body. As such, the ENS is best adapted to coordinate physiologically related responses between different cell types and propagate them over vast regions of the GI tract. The cellular biology of the GI tract is inherently complex, necessitating the development and testing of interdisciplinary hypotheses, a challenge with immense implications for human physiology.

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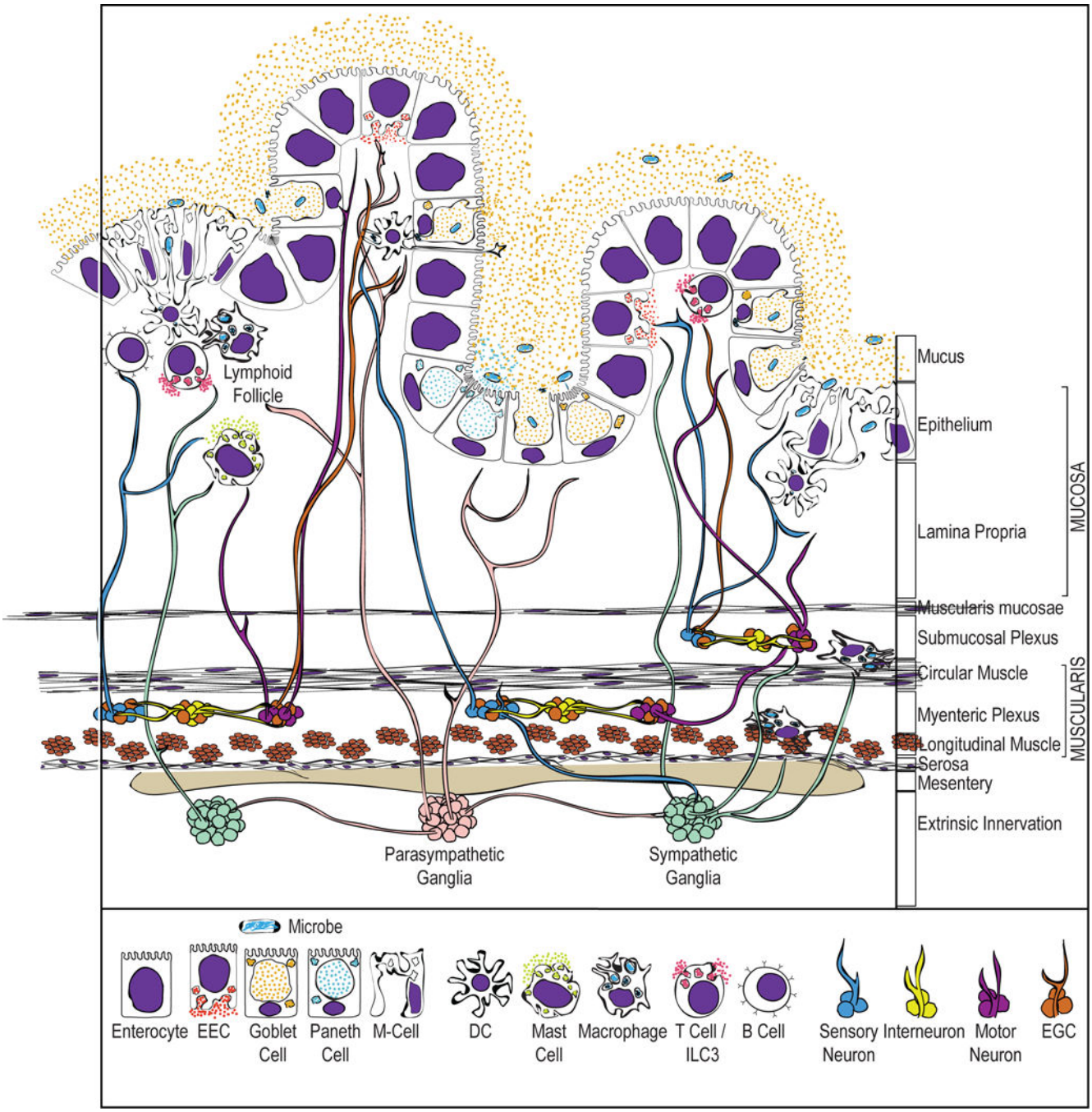
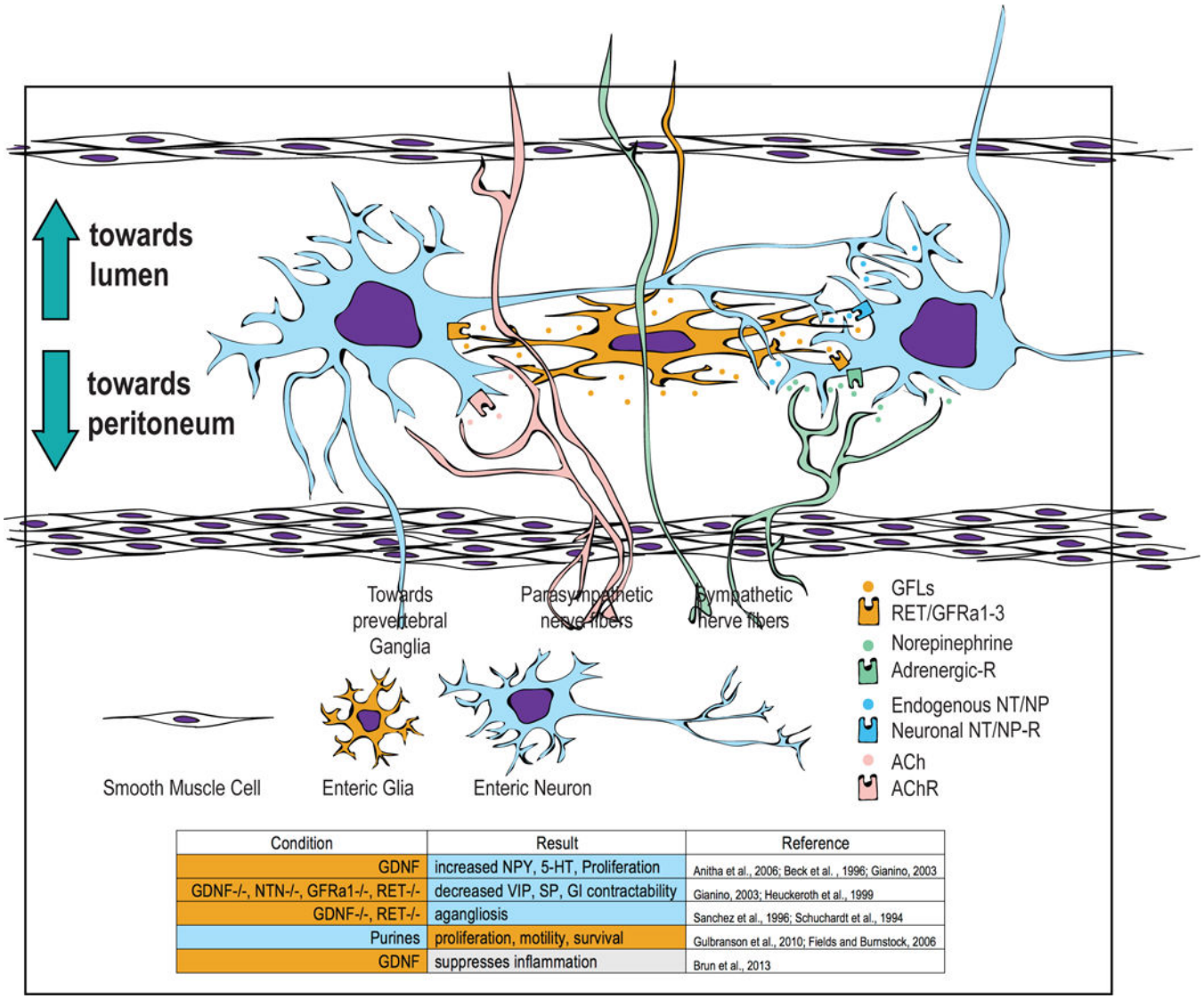


Figure 1. Anatomy of the GI Tract

The GI tract is comprised of distinct cross-sectional compartments. Extrinsic, sympathetic and parasympathetic nerve fibers enter the GI tract through the mesentery and can extend throughout all layers of intestinal tissue. The myenteric and submucosal plexuses form the dense nerve network that innervates the entire length and depth of the GI tract. Various immune cells are resident to the muscularis, but are also highly abundant in the lamina propria, especially in Peyer's patches and lymphoid follicles. These immune cells are also in close proximity to neurons and glia. The epithelium shown here is made up of 5 different

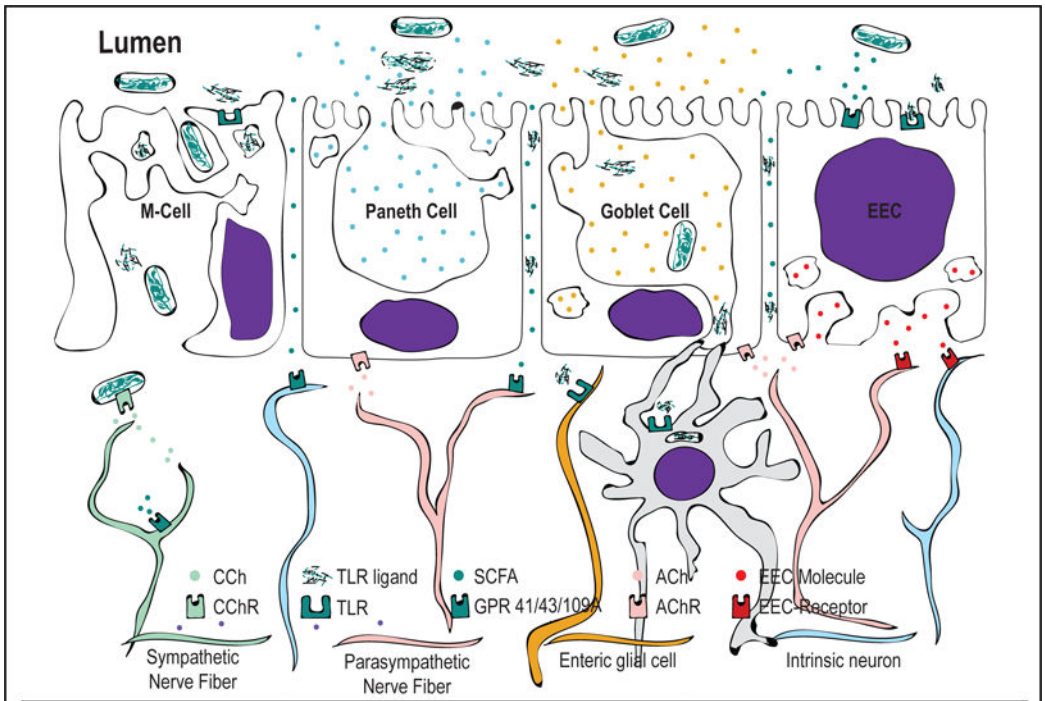
cell types. These include absorptive enterocytes, EECs, goblet cells, Paneth cells, and M-cells.



Condition	Result	Reference
GDNF	increased NPY, 5-HT, Proliferation	Anitha et al., 2006; Beck et al. , 1996; Gianino, 2003
GDNF-/-, NTN-/-, GFRa1-/-, RET-/-	decreased VIP, SP, GI contractability	Gianino, 2003; Heuckeroth et al., 1999
GDNF-/-, RET-/-	agangliosis	Sanchez et al., 1996; Schuchardt et al., 1994
Purines	proliferation, motility, survival	Gulbranson et al., 2010; Fields and Burnstock, 2006
GDNF	suppresses inflammation	Brun et al., 2013

Figure 2. Connectivity of Enteric Neurons and Glia

Enteric neurons are located in either the submucosal or myenteric plexuses. Both plexuses are located in between two muscle layers (see Figure 1). Parasympathetic nerve fibers release acetylcholine and sympathetic nerves release norepinephrine. These extrinsic nerve fibers can innervate enteric neurons but also associate with the cells in the smooth muscle, lamina propria, and the epithelium. Enteric neurons can innervate one another or extend towards the lamina propria, and specific intestinofugal (IFANs) can synapse onto sympathetic ganglia. Enteric glial cells make and release neurotrophic factors, associate with enteric neurons, and extend throughout the mucosa. The left and middle columns are color coded to represent cells and molecules that generate specific conditions and the results that are produced from those conditions, respectively.

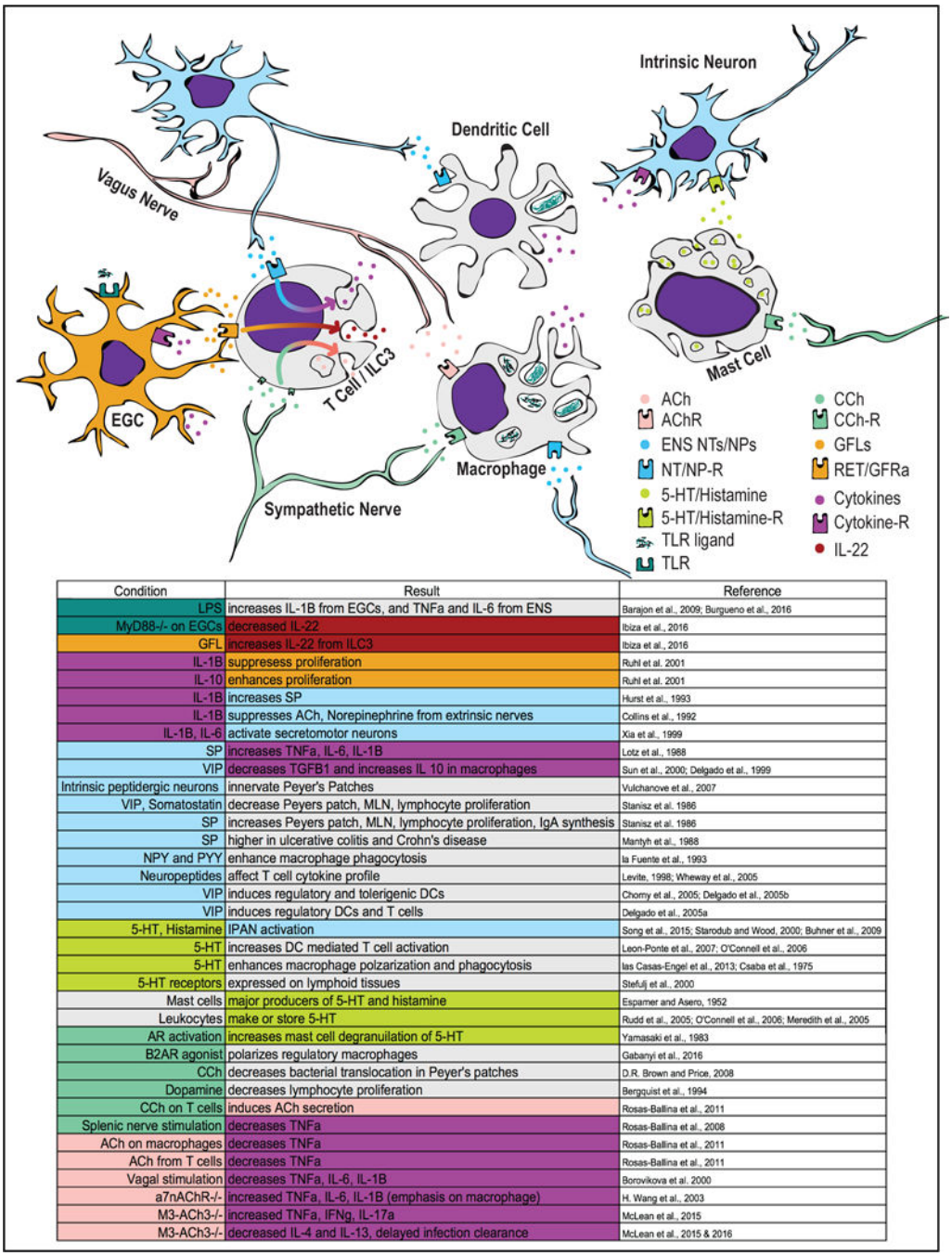


Condition	Result	Reference
mAChR agonist bethanechol	increases Paneth cell degranulation	Satoh et al. 1992
mAChR antagonist atropine	decreases Paneth cell degranulation	Satoh, 1988
AChR agonist carbahol	increases Goblet cell mucin secretion	Gustafsson et al., 2012; Birchenough et al., 2016
AChR agonist carbahol	increases Goblet cell associated passages (GAPs)	McDole et al. 2012
mAChR agonist bethanechol	increases EEC secretion	Anini and Brubaker, 2003
GF Mice	less neurons and decreased ENS excitability	Collins et al., 2014; McVey Neufeld et al., 2013
Antibiotics	decreases motility and GI sensitivity to ACh	Grasa et al., 2015
TLR4-/-	fewer neurons in ENS	Anitha et al., 2012
Butyrate	Activates intrinsic ENS	Neunlist and Dobreva, 1999
Butyrate	increases cholinergic neurons in myenteric plexus	Soret et al., 2010
L. reuteuri	increased IPAN excitability	Kunze et al., 2009
L. rhamnosus	lowers anxiety via vagus nerve, increases vagal firing	Bravo et al., 2011; Perez-Burgos et al., 2013
B. fragilis PSA	increased IPAN excitability	Mao et al., 2013
Microbiota, *Clostridia*, GUS enzyme	increases CCh availability	Asano et al., 2012
CCh	enhances *E. coli* and *Salmonella* virulence	Njoroge and Sperandio, 2012; Moreira and Spreandio, 2012
DBH-/-	suppressed *C. rodentium* infection	Moreira et al., 2016
Microbiota	increases EGC growth	Kabouridis et al., 2015
Antibiotics	decreases GDNF, GFRa1, RET	Brun et al., 2013
TLR2-/-	decreased GDNF, RET	Brun et al., 2013
TLR-ligands	increase GDNF	Brun et al., 2013
Clostridia, SCFAs	5-HT from enterochromaffin cells	Yano et al., 2015; Reigstad et al., 2015
GPR 41, GPR43 activation	PYY and GLP-1 release	Psichas et al., 2014; Nohr et al., 2013
GLP-1, CCK	Activate ENS	Amato et al., 2010; Ngu, 1985
Butyrate	decreases monocyte activation and mast cell degranulation	Digby et al., 2012; Diakos et al., 2006

Figure 3. Interactions at the Intestinal Epithelium

The intestinal epithelium is where luminal constituents are actively or passively transported into the tissue. Extrinsic nerves and neurons are found near the epithelium, and thus the molecules that cross the epithelium and those that are secreted basolaterally can potentially have an effect on their activity. Microbes or microbial parts can cross the epithelium and affect other cell types through M-cells, immunoglobulin-mediated transcytosis, goblet cell-associated passages (GAPs), and by general leakiness of the epithelium. Dendritic cells (DCs) and macrophages can phagocytose microbial antigens and secrete cytokines that can

have an effect on neurons as well. Parasympathetic fibers release acetylcholine (ACh) and induce secretion of intracellular stores of molecules. In goblet cells, ACh also increases rates of DC luminal sampling via GAPs. EECs can also release neuroendocrine molecules in response to TLR stimulation and SCFAs. These molecules released basolaterally can potentially regulate the activity of neurons. Enteric glial cells can also project towards the epithelium, potentially allowing microbes to impact their function. Enteric neurons can be activated by commensal and pathogenic bacteria, as well as short chain fatty acids that diffuse across the epithelium. The left and middle columns are color coded to represent cells and molecules that generate specific conditions and the results that are produced from those conditions, respectively.



Condition	Result	Reference
LPS	increases IL-1B from EGCs, and TNFa and IL-6 from ENS	Barajon et al., 2009; Burgueno et al., 2016
MyD88-/- on EGCs	decreased IL-22	Ibiza et al., 2016
GFL	increases IL-22 from ILC3	Ibiza et al., 2016
IL-1B	suppresess proliferation	Ruhl et al. 2001
IL-10	enhances proliferation	Ruhl et al. 2001
IL-1B	increases SP	Hurst et al., 1993
IL-1B	suppresses ACh, Norepinephrine from extrinsic nerves	Collins et al., 1992
IL-1B, IL-6	activate secretomotor neurons	Xia et al., 1999
SP	increases TNFa, IL-6, IL-1B	Lotz et al., 1988
VIP	decreases TGFB1 and increases IL 10 in macrophages	Sun et al., 2000; Delgado et al., 1999
Intrinsic peptidergic neurons	innervate Peyer's Patches	Vulchanova et al., 2007
VIP, Somatostatin	decrease Peyers patch, MLN, lymphocyte proliferation	Stanisz et al. 1986
SP	increases Peyers patch, MLN, lymphocyte proliferation, IgA synthesis	Stanisz et al. 1986
SP	higher in ulcerative colitis and Crohn's disease	Mantyh et al., 1988
NPY and PYY	enhance macrophage phagocytosis	la Fuente et al., 1993
Neuropeptides	affect T cell cytokine profile	Levite, 1998; Wheway et al., 2005
VIP	induces regulatory and tolerigenic DCs	Chorny et al., 2005; Delgado et al., 2005b
VIP	induces regulatory DCs and T cells	Delgado et al., 2005a
5-HT, Histamine	IPAN activation	Song et al., 2015; Starodub and Wood, 2000; Buhner et al., 2009
5-HT	increases DC mediated T cell activation	Leon-Ponte et al., 2007; O'Connell et al., 2006
5-HT	enhances macrophage polzarization and phagocytosis	las Casas-Engel et al., 2013; Csaba et al., 1975
5-HT receptors	expressed on lymphoid tissues	Stefulj et al., 2000
Mast cells	major producers of 5-HT and histamine	Espamer and Asero, 1952
Leukocytes	make or store 5-HT	Rudd et al., 2005; O'Connell et al., 2006; Meredith et al., 2005
AR activation	increases mast cell degranuilation of 5-HT	Yamasaki et al., 1983
B2AR agonist	polarizes regulatory macrophages	Gabanyi et al., 2016
CCh	decreases bacterial translocation in Peyer's patches	D.R. Brown and Price, 2008
Dopamine	decreases lymphocyte proliferation	Bergquist et al., 1994
CCh on T cells	induces ACh secretion	Rosas-Ballina et al., 2011
Splenic nerve stimulation	decreases TNFa	Rosas-Ballina et al., 2008
ACh on macrophages	decreases TNFa	Rosas-Ballina et al., 2011
ACh from T cells	decreases TNFa	Rosas-Ballina et al., 2011
Vagal stimulation	decreases TNFa, IL-6, IL-1B	Borovikova et al. 2000
a7nAChR-/-	increased TNFa, IL-6, IL-1B (emphasis on macrophage)	H. Wang et al., 2003
M3-ACh3-/-	increased TNFa, IFNg, IL-17a	McLean et al., 2015
M3-ACh3-/-	decreased IL-4 and IL-13, delayed infection clearance	McLean et al., 2015 & 2016

Figure 4. Interactions between GI Immune Cells and the ENS

Extrinsic nerves, intrinsic neurons, and enteric glial cells are in close proximity to each other and to the immune cells in the GI tract. Thus, the molecules that are produced by one cell can have an effect on another cell, given that the latter expresses a receptor to recognize the molecule. By these parameters, interactions between neurons/glia and immune cells are, in theory, abundant, and some of these putative interactions are presented here. Immune cells can be influenced by neurotransmitters and neuropeptides produced by intrinsic neurons of the ENS (as well as those produced by extrinsic nerve fibers), and cytokines produced by

immune cells can have a reciprocal effect on neurons. The left and middle columns are color coded to represent cells and molecules that generate specific conditions and the results that are produced from those conditions, respectively.